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Bacillus anthracis Multiplication, Persistence, and Genetic Exchange in the Rhizosphere of Grass Plants

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***Bacillus anthracis*, the causative agent of anthrax, is known for its rapid proliferation and dissemination in mammalian hosts. In contrast, little information exists regarding the lifestyle of this important pathogen outside of the host. Considering that *Bacillus* species, including close relatives of *B. anthracis*, are saprophytic soil organisms, we investigated the capacity of *B. anthracis* spores to germinate in the rhizosphere and to establish populations of vegetative cells that could support horizontal gene transfer in the soil. Using a simple grass plant-soil model system, we show that *B. anthracis* strains germinate on and around roots, growing in characteristic long filaments. From 2 to 4 days postinoculation, approximately one-half of the *B. anthracis* CFU recovered from soil containing grass seedlings arose from heat-sensitive organisms, while *B. anthracis* CFU retrieved from soil without plants consisted of primarily heat-resistant spores. Coinoculation of the plant-soil system with spores of a fertile *B. anthracis* strain carrying the tetracycline resistance plasmid pBC16 and a selectable *B. anthracis* recipient strain resulted in transfer of pBC16 from the donor to the recipient as early as 3 days postinoculation. Our findings demonstrate that *B. anthracis* can survive as a saprophyte outside of the host. The data suggest that horizontal gene transfer in the rhizosphere of grass plants may play a role in the evolution of the *Bacillus cereus* group species.**

Members of the genus *Bacillus* are among the most common organisms isolated from soil. This heterogeneous taxonomic group of endospore-forming bacteria contains more than 65 species, and new members are being described continually (13, 16, 27). The physiological and metabolic versatility of *Bacillus* species permits rapid germination of spores when nutrients become available (55). The genus is comprised primarily of saprophytic organisms, and only three species are known to cause disease in mammals: *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis*. Based on DNA hybridization and 16S rRNA sequences (49), these three taxa plus *B. mycoides* form the *B. cereus* group of species (43). *B. thuringiensis* produces delta endotoxins and is primarily an insect pathogen (52). However, *B. thuringiensis* strains can cause wound infections after severe trauma (26). *B. cereus* is an opportunistic pathogen that can cause nongastrointestinal infections in postsurgical patients and eye infections (5), as well as produce toxins associated with emetic and diarrheal food poisoning (14, 36). *B. anthracis* is the etiologic agent of anthrax (42, 61).

B. anthracis has a salient position within the group, as it is considered the only "obligate pathogen" of the genus *Bacillus* because it is thought to multiply almost exclusively in the animal host (61). While all mammals and several avian species are known to be susceptible to the disease, anthrax affects mainly wild and domesticated herbivores. Sporadic occurrences of anthrax are reported regularly worldwide, and the disease is enzootic in most African and Asian countries, parts of Europe, the Americas, and Australia (62). Control programs, including immunization with the live Sterne spore vaccine (56, 57), have led to a global decline in livestock cases, but the disease remains unchecked in wildlife in many parts of the world (29).

The ecological cycle of *B. anthracis* is centered around the metabolically dormant spore (31, 42, 59, 61). In nature, herbivores ingest and/or inhale spores from soil while grazing. The spores enter the pharyngeal or intestinal mucosa via lesions caused by sharp-edged particles, grit, spiky leaves or thorns in the feed, and initiate infection (61). Carnivores, such as cheetahs, jackals, hyenas, wild dogs, and scavenging vultures, ingest vegetative bacilli and spores with the meat of infected dead or dying animals. Disease incidence in these carnivorous species is highly variable. According to the current model for pathogenesis, once inside the host, spores are phagocytosed and transported to regional lymph nodes. After spore germination, vegetative bacilli multiply rapidly in the blood, cerebrospinal fluid, and other tissues, producing an antiphagocytic capsule and the anthrax lethal and edema toxins (38, 41, 42). Blood from carcasses can contain up to 10^9 CFU of *B. anthracis* per ml. The organisms enter soil and water during terminal hemorrhaging or upon carcass destruction by scavenging carnivores (37). Since sporulation requires oxygen, spore formation begins in pools of blood and tissue fluids around a carcass (60). Numbers of *B. anthracis* CFU from soil and water samples around carcass sites are highly variable and decline rapidly to levels below the threshold of detection. The rapid decline may be attributed to efficient dispersal by carnivorous mammals, vultures, insects, rainwater, and wind (37, 59, 61) and/or to relatively low viability of *B. anthracis* vegetative cells and spores in the environment (37).

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In light of these observations, and in the absence of transmission between herbivores, it is difficult to explain large outbreaks of the disease in livestock and wild animal herds that arise and reoccur in time intervals ranging from years to decades. The infective dose for large herbivores in the wild is not known. Experimental infections of animals indicate that the spore doses required to kill 50% of the animals tested vary widely in different species (12, 15, 61). If *B. anthracis* replicates only in host animals, carcass sites serve as the primary point sources for infective spores. These sites may be the only sources of spores in high enough numbers to cause disease.

Patterns of certain epizootics can be explained more easily when it is postulated that low levels of *B. anthracis* contamination persist in the soil and that outbreaks occur following bursts of spore germination and multiplication of bacilli under favorable environmental conditions (32). It has been hypothesized that before infection of livestock occurs, *B. anthracis* propagates in soil in locations termed "incubator areas." According to Van Ness, soils rich in calcium and organic matter with a pH above 6.0 and soil temperatures above 15°C favor multiplication of bacilli in topographically well-defined areas in which animals become infected and outbreaks of anthrax disease occur (63).

Despite these insights, the growth of vegetative *B. anthracis* outside of the host in incubator areas or any other locations in the environment has not been reported. It is notable that factors cited in explanations of geographical distribution and seasonality of anthrax outbreaks in herbivores, such as climate, geology, soil properties, and movement of water and cattle herds, also affect the plants on which animals feed. While *B. cereus* is known to colonize plant roots (14, 17, 20, 33, 39, 40, 53, 58) the possibility that *B. anthracis* spores could respond to germinants in the immediate vicinity of plant roots, i.e., the rhizosphere (9), has not been considered. Nutrients in the rhizosphere could sustain the multiplication of vegetative *B. anthracis* in this ecological niche, potentially amplifying the primary inoculum of spores stemming from a carcass. To test some aspects of this hypothesis, we developed a plant-soil model system to address fundamental questions regarding the behavior of *B. anthracis* in the rhizosphere of grasses.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists strains and plasmids used in this study. Plasmid pUTE610 carries the gene for green fluorescent protein (GFP) *gfp*mut3a (8, 17) fused to a 645-bp PCR product containing the constitutively active promoter of the *B. anthracis* metalloprotease gene, *inhA* (BA1295). The PCR employed template DNA from *B. anthracis* 7702 and oligonucleotide primers ES26 (5′-ATTCTAGACCCAGGATTTTCACCCTTTT-3′), containing an XbaI site (underlined), and ES44 (5′-CGGGATCCTTCTTGATCCAATGCGT CAG-3′), containing a BamHI site (underlined). The PCR product was ligated into pGEM-T Easy (Promega, Madison, WI), released by digestion with BamHI and XbaI, and ligated into the same sites of pAD123 (17) upstream of the promoterless *gfp*mut3a gene. Digestion of the construct with SacI and HindIII released the *inhAp*::*gfp*mut3a fusion for subcloning into shuttle vector pUTE568 (7).

A *B. anthracis* strain carrying a selectable marker in a nonessential chromosomal locus was used to facilitate recovery of bacteria from soil. The *B. anthracis plcR* locus has been reported to encode a nonfunctional protein (1, 54); 353 nucleotides (nt) of the *plcR* coding sequence (AF132086) of strain 7702 were replaced with an Ω spectinomycin resistance cassette (Ω*sp*) (50) using a protocol described previously (10). Briefly, the gene replacement vector contained the Ω*sp* cassette flanked by DNA sequences corresponding to regions upstream and downstream of the target site. Flanking sequences were amplified using the following primer pairs: ES40 (5′-AACTGCAGTGTTGCGGAAACGTTAAAG A-3′), containing a PstI site (underlined), and ES41 (5′-CGGGATCCCGATT CAATTCGGCTCACTT-3′), containing a BamHI site (underlined), for the upstream flanking sequence and ES 42 (5′-CGGGATCCTTGAAAACGCAATT GCAAAC-3′), with a BamHI site (underlined), and ES43 (5′-ACGCGTCGAC TCGTATCTCCTGCCCAATTC-3′), with a SalI site (underlined), for the downstream flanking sequence. Double-crossover recombination yielded mutant UT258, in which *plcR* sequences from 116 nt downstream of the translational start codon to 172 nt upstream of the translational stop codon were replaced with Ω*sp*.

Electroporation of *B. anthracis* and extraction of plasmid DNA were performed as described elsewhere (34).

TABLE 1. Strains and plasmids used in this study

Strain/plasmid	Relevant characteristic(s)	Reference
Strains		
7702	pXO1$^+$	6
UT258	7702 containing *plcR*::Ω*sp*; Spc^r	This work
UM44-1tr203-1	pXO1$^+$ pXO12$^+$ pBC16$^+$ Str^r Ind$^-$ Tra$^+$ Tc^r	3
UM23C1-2	Rif^r Ura$^-$	3
Plasmids		
pAD123	Promoterless *gfp*mut3a; Ap^r Cm^r	17
pUTE568	Constructed from pBC16 and pBC; Cm^r in *E. coli*; Em^r in *B. anthracis*	7
pUTE610	*inhAp*::*gfp*mut3a; Cm^r in *E. coli*; Em^r in *B. anthracis*	This work

Media and bacterial culture conditions. *Escherichia coli* and *B. anthracis* were grown in Luria-Bertani (LB) broth (BD Difco, Franklin Lakes, NJ) at 37°C. Plasmid DNA was isolated from *B. anthracis* strains following culture in brain heart infusion medium (BD Difco). For production of electrocompetent *B. anthracis* cells, cultures were grown in brain heart infusion medium containing 0.5% glycerol. Min IC medium (3) containing uracil (2.5 mg/ml) or tryptophan (5 mg/ml) was used to confirm the genetic background of transcipient isolates. When appropriate, media contained ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), erythromycin (5 μg/ml), rifampin (10 μg/ml), spectinomycin (100 μg/ml), streptomycin (200 μg/ml), tetracycline (5 μg/ml), or cycloheximide (50 μg/ml).

Plant-soil system and growth conditions. The substrate was potting mix composed of processed pine bark, sphagnum peat, perlite, and lime (Teas Nursery Co. Inc., Houston, TX). Prior to the planting of germinated seeds, the substrate was autoclaved to reduce the viable-organism content, and calcium oxide was added to raise the pH of a suspension in water to pH 8. Tall fescue (*Festuca arundinacea*) seeds (BWI Companies, Inc., Texarkana, TX) were germinated for 4 to 5 days on water agar at 30°C in the dark until roots emerged. Seeds were then transferred in groups of three into 1.5-ml Eppendorf tubes containing 0.75 ml air-dried substrate. Following inoculation with a 10-μl *B. anthracis* spore suspension and a 10-min incubation on the bench top, 200 μl of sterile water was added to each tube, and the tubes were incubated in a growth chamber at 30°C with a 14-h light/10-h dark cycle. The plants were watered daily with autoclaved tap water.

Fluorescence microscopy. Plant seedlings were removed from the tubes and placed in dishes of sterile water. Roots were separated from soil by probing them gently with forceps. Monoclonal antibody generated against cell wall polysaccharide antigen and conjugated to Alexa Fluor 555 (Molecular Probes, Eugene, OR) was added directly to the roots in 10 mM phosphate-buffered saline, 0.3% Tween 20, pH 7.2, on microscope slides. After incubation for 15 min at room temperature in the dark, the roots were rinsed with 500 μl 10 mM phosphate-buffered saline, 0.3% Tween 20, pH 7.2; covered with a coverslip; and visualized using rhodamine and fluorescein isothiocyanate (FITC) filter sets on a Nikon Eclipse E600 microscope. Seedlings inoculated with a droplet of sterile water served as controls.

Recovery of heat-resistant and heat-sensitive *B. anthracis* CFU from the plant-soil system. Tubes containing soil only or soil with plants were inoculated with spore suspensions of the spectinomycin-resistant strain UT258. Inoculum concentrations were determined by spread plating dilutions on LB agar containing spectinomycin. CFU were recovered on days 0 (following a 30-min incubation in the growth chamber), 2, 4, and 16 as follows. For tubes containing plants, leaves were cut at the tube opening and discarded. Plants (shoots and roots) were removed from the soil and transferred to tubes containing 400 μl sterile 10 mM

phosphate-buffered saline, pH 7.2. To the remaining soil, the appropriate amount of sterile tap water was added to allow retrieval of no more than 600 μl of liquid. Water and soil were vortexed for 5 min at the highest setting. All liquid from the tubes was removed and added to the corresponding plant in 400 μl phosphate-buffered saline. Tubes containing soil without plants were processed similarly so that 1-ml suspensions were obtained. The suspensions were vortexed for 30 s and, following removal of the plant, were divided into two 500-μl portions. One portion was held at 65°C for 30 min. The other portion remained at room temperature. Suspensions were diluted in sterile water, plated on LB agar containing cycloheximide and spectinomycin, and incubated overnight at 37°C to determine numbers of heat-sensitive and heat-resistant CFU. Differences between the means of CFU were tested for significance by analysis of variance.

Coinoculation with donor and recipient strains and recovery of transcipients. Inoculum concentrations were determined by dilution plating on LB agar containing tetracycline (5 μg/ml) for donor strain UM44-1tr203-1 and rifampin (10 μg/ml) for recipient strain UM23C1-2. Each of 10 tubes containing freshly transplanted seedlings and soil and 10 tubes containing soil only were coinoculated with donor and recipient strains by placing 20-μl droplets of a 1:1 mixture of the spore suspensions on the air-dried soil. In addition, four tubes with plants were inoculated with donor spores only and four tubes with plants were inoculated with recipient spores only. After the addition of 200 μl of sterile water, all tubes were placed in the growth chamber.

Three days postinoculation, *B. anthracis* CFU were recovered as described above. Suspensions were not heated before being plated on selective media. To determine the recovery of donor and recipient strains, suspensions from tubes inoculated with the donor only were plated on LB agar with cycloheximide and tetracycline and suspensions from tubes inoculated with the recipient only were plated on LB agar with cycloheximide and rifampin. To test for spontaneous tetracycline (Tc^r) or rifampin (Rif^r) resistance, two 350-μl aliquots of undiluted donor or recipient suspensions were plated on LB agar with cycloheximide, tetracycline, and rifampin.

Transcipients were identified as Tc^r Rif^r CFU isolated from plant-soil systems coinoculated with the donor and recipient. Suspensions from these tubes were plated undiluted in 300-μl aliquots on LB medium with cycloheximide, tetracycline, and rifampin. Colonies were streaked to selective media to confirm the antibiotic resistance phenotype and subsequently plated on Min IC with and without uracil and with and without tryptophan to confirm the Ura^- genetic background of the recipient strain UM23C1-2. Plasmid DNA isolated from transcipients was digested with BamHI and subjected to agarose gel electrophoresis to verify the presence of pBC16 (24). The presence of plasmid-specific sequences was also assessed using PCR. DNA corresponding to the *tet* gene of pBC16 was amplified using primers 5′-CAAATATAATTCCCCCACAA-3′ and 5′-TTAATAAACCACCTGCGAGT-3′. Primers 5′-TGTTCAAAAAGGAAAAGGAA-3′ and 5′-GTATTTGGATGACGCTGTTT-3′ were used to amplify DNA of the *atxA* gene on pXO1. DNA corresponding to the internal resolution site of Tn*4430* on the fertility plasmid pXO12 (25) was amplified using primers described previously (51).

RESULTS

***B. anthracis* spores germinate in the rhizosphere of grass plants.** Visualization of vegetative *B. anthracis* in the plant-soil system was accomplished using *B. anthracis* mutants expressing GFP and by employing an Alexa Fluor 555-conjugated monoclonal antibody raised against *B. anthracis* cell wall galactose-*N*-acetylglucosamine-polysaccharide (21). GFP-synthesizing strains harbored the high-copy-number plasmid pUTE610 containing *gfp*mut3a (8, 17, 18) under the control of the constitutive *B. anthracis* promoter *inhAp*. In *B. thuringiensis*, *inhA* encodes a metalloprotease that hydrolyzes cecropins and attacins in the immune hemolymph of *Hyalophora cecropia* (11, 19, 22, 23). The function of *inhA* in *B. anthracis* is not clear.

Vegetative cells and spores of 7702(pUTE610) and UT258 (pUTE610) appeared brightly fluorescent, allowing detection of both forms of the bacterium in the plant-soil system. Green-fluorescent cells were distinguished from green-fluorescent spores by their filamentous growth and, especially in the case of vegetative cells, which grew as single rods, by the labeled monoclonal antibody, which reacted against cells but did not bind to dormant spores (21).

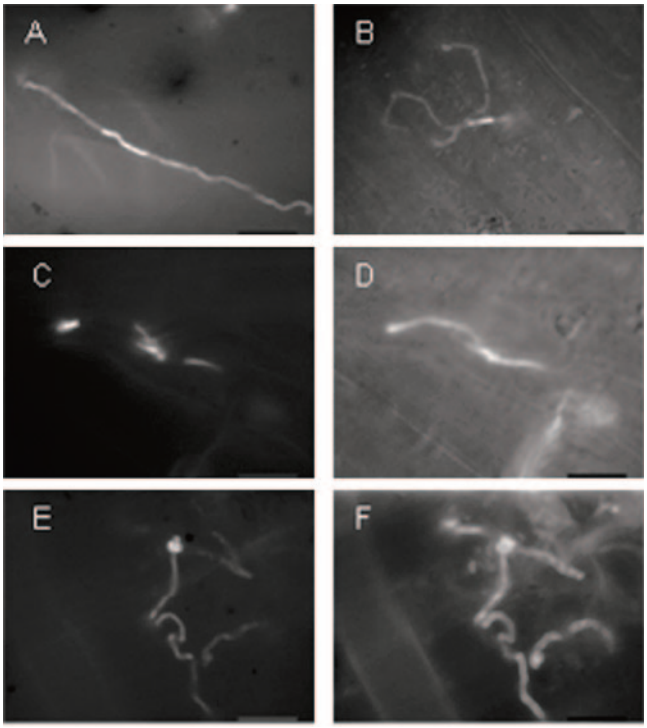

FIG. 1. *B. anthracis* on fescue roots. Following inoculation of the soil with *B. anthracis* spores, green-fluorescent vegetative cells were found in the rhizosphere growing in typical chains. (A) UT258(pUTE610), day 6, FITC. (B) UT258(pUTE610), day 6, FITC. Staining with the red-fluorescent anti-cell wall polysaccharide antibody revealed that not all of the cells in a chain expressed GFP. (C) 7702(pUTE610), day 3, FITC. (D) The same field as in panel C but viewed with a tetramethyl rhodamine isothiocyanate (TRITC) filter. The cells were labeled with Alexa Fluor 555-conjugated anti-polysaccharide monoclonal antibody. Green-fluorescent chains of *B. anthracis* vegetative cells were detected after more than 6 weeks of incubation. (E) UT258(pUTE610), day 46, FITC. (F) The same field as in panel E but viewed with a TRITC filter. The cells were labeled with Alexa Fluor 555-conjugated anti-polysaccharide monoclonal antibody. Bars = 50 μm.

Freshly transplanted tall fescue seedlings were inoculated with 10^6 spores of 7702(pUTE610) or UT258(pUTE610), and the association of *B. anthracis* with plant roots was monitored for up to 7 weeks. Microscopic inspection on the first few days following inoculation showed green-fluorescent vegetative *B. anthracis* in the rhizosphere (Fig. 1A and B). Different growth phenotypes, such as short corkscrew-like and warped chains versus long straight chains, were detected easily for up to 2 weeks postinoculation. Staining with the fluorescent anti-cell wall polysaccharide antibody revealed that not all of the cells in a chain expressed GFP (Fig. 1C and D). Although the ability to detect green-fluorescent chains of cells in the rhizosphere diminished with time, green-fluorescent chains and webs of vegetative *B. anthracis* filaments were detected after more than 6 weeks of incubation (Fig. 1E and F). Populations of single rod-shaped *B. anthracis* cells could be identified as vegetative cells after double labeling them with the anti-cell wall polysaccharide antibody and thus could be differentiated from green-

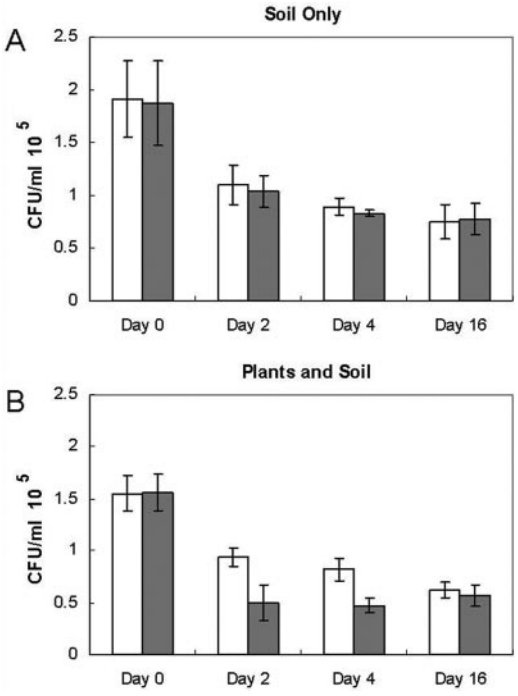

FIG. 2. CFU recovered from tubes inoculated with 1.3×10^5 spores of UT258 on the days shown. The open bars show total viable-cell counts. The filled bars show spores (CFU isolated following heat treatment). Each bar represents the average number of CFU retrieved from seven tubes containing either soil (A) or soil and seedlings (B). The error bars represent ±1 standard deviation.

fluorescent, ungerminated *B. anthracis* spores (data not shown). Green-fluorescent bacteria were never observed on control plants, which were inoculated with water only.

Quantitative analysis of *B. anthracis* multiplication and persistence in the rhizosphere. Microscopic detection of vegetative *B. anthracis* on seedling roots provided the framework for an experimental timeline in which to quantify *B. anthracis* CFU recovered from plant roots and soil. We determined the numbers of viable spores and vegetative cells retrieved from plant roots and soil on days 0, 2, 4, and 16 following inoculation with UT258. The spectinomycin resistance marker of this strain facilitated selective recovery from soil and plant material.

The overall recovery of spectinomycin-resistant *B. anthracis* CFU was determined in three experiments whose designs differed only in the amount of inoculum added on day 0 (1.2×10^4, 1.3×10^5, or 1.4×10^6 CFU per tube). In the representative experiment shown in Fig. 2, each tube was inoculated with 1.3×10^5 CFU. The average numbers of spores retrieved on day 0 were sometimes up to 46% higher than the viable counts of the inoculum itself. This difference most likely resulted from dispersal of spore clumps in the inoculum following vortexing with soil particles. Recovery decreased over time from complete retrieval of the inoculum 30 min after inoculation on day 0 to about 50% retrieval (0.6×10^5 to 0.8×10^5 CFU/tube) on day 16. The largest decline was usually found between day 0 and day 2. The decrease in numbers may reflect death of the bacteria and/or tight association of the bacteria with soil particles and plant material following incubation.

In all three experiments, for the tubes containing soil only, the average number of CFU from heat-resistant organisms did not differ significantly from the average total number of CFU, indicating that at every sampling date the *B. anthracis* CFU retrieved arose almost exclusively from spores (Fig. 2A). In all experiments for tubes containing plants, on days 2 and 4 there was a statistically significant difference ($P = 0.00006$ and $P = 0.00001$, respectively, for the experiment shown in Fig. 2) between the average total number of CFU retrieved and the average number of heat-resistant bacteria retrieved from the tubes (Fig. 2B). On days 2 and 4, 47% and 43% of the total CFU recovered represented heat-sensitive organisms, indicating a high number of vegetative cells in tubes containing plants shortly after inoculation. However, by day 16, the number of CFU derived from heat-sensitive CFU dropped dramatically to 8% of the total CFU recovered. Given the microscopic observations indicating long chains of vegetative cells in the plant-soil system, the data from CFU determinations underestimated the number of vegetative *B. anthracis* cells present in the system. Taken together, the data suggest that the presence of plant roots enhanced germination and vegetative growth of *B. anthracis* for a period of several days.

Transfer of pBC16 between *B. anthracis* strains during growth in the rhizosphere. Certain *B. thuringiensis* plasmids are self-transmissible and can promote intra- and interspecies transfer of other plasmids among *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains during coculture in rich laboratory media (3, 25, 47). The presence of vegetative *B. anthracis* in the rhizosphere led us to hypothesize that the metabolic activity of *B. anthracis* in the plant-soil system is sufficient to permit plasmid transfer between *B. anthracis* strains.

B. anthracis strain UM44-1tr203-1 was used as a donor strain. The strain carries the *B. anthracis* virulence plasmid pXO1; the *B. cereus* plasmid pBC16, which confers tetracycline resistance (3); and *B. thuringiensis* plasmid pXO12, which encodes δ-endotoxin. Plasmid pXO12 is a fertility plasmid and is capable of self-transfer and mobilization of other plasmids, including pBC16, into *B. anthracis*, *B. cereus*, and *B. thuringiensis*. Plasmid pXO12-mediated transfer of chromosomal DNA has not been demonstrated (3). The cured strain *B. anthracis* UM23C1-2 served as the recipient. This strain is Rif^r and lacks pXO1. It carries neither pBC16 nor pXO12 (3) (Table 1). Experiments were designed to select for transcipients which were resistant to both rifampin and tetracycline.

Three experiments were carried out in which 6×10^4 to 2×10^5 spores of the donor strain mixed with 2×10^5 to 3×10^5 spores of the recipient strain were added to tubes containing soil only or soil and seedlings. Three days after inoculation, the numbers of Tc^r Rif^r transcipient CFU were determined for each tube. No Tc^r Rif^r isolates were recovered from tubes that contained soil without plants. In contrast, Tc^r Rif^r transcipients were obtained from tubes containing grass seedlings, as shown in Fig. 3. In experiment 1, transcipients were isolated from 9 of the 10 tubes containing plants. In the following two experiments, 4 out of 10 tubes (experiment 2) and 10 out of 10 tubes (experiment 3) yielded transcipients. The numbers of transcipient CFU per tube were highly varied, ranging from 1 to 46. All Tc^r Rif^r transcripients were tested for the ability to grow on media containing or lacking uracil and/or tryptophan. All isolates were uracil auxotrophs, corresponding to the genetic background of the recipient strain. To determine retrieval ef-

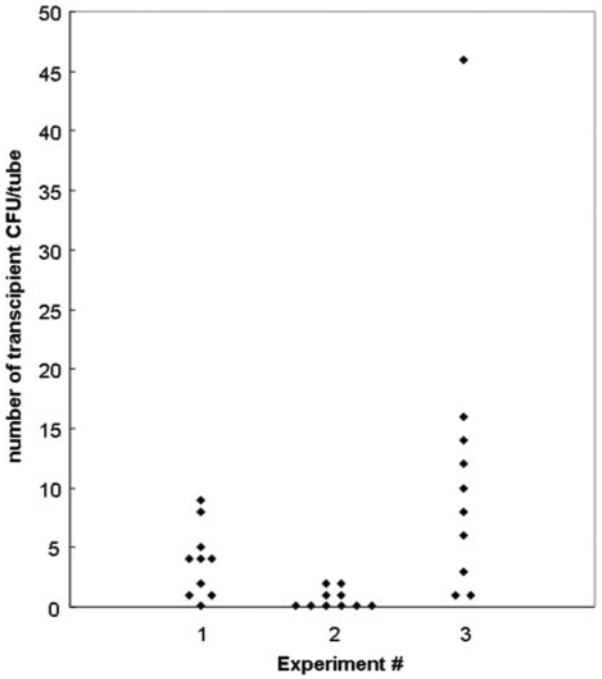

FIG. 3. Transcipient CFU recovered from tubes containing plants and soil 3 days after coinoculation with a *B. anthracis* donor and a recipient strain. In each experiment, 10 tubes containing grass plants and soil were assayed. Each data point represents the number of transcipients isolated from 1 of the 10 tubes. No transcipients were detected in tubes containing soil only (data not shown).

ficiency and to test for spontaneous rifampin and tetracycline resistance in the donor and recipient, respectively, CFU were recovered from tubes which had been inoculated with only one strain. Retrieval efficiencies ranged from 52% to 89%, and spontaneous antibiotic resistance was not observed.

The genetic content of representative transcipients was determined using plasmid DNA extraction and PCR amplification of plasmid-specific sequences. The presence of the tetracycline resistance gene was confirmed for at least one transcipient isolate from every tube using PCR (Fig. 4, top). All the transcipients tested carried the *tet* gene. In addition, the presence of pBC16 in several transcipient strains, representative of each of the three different coinoculation experiments, was confirmed following restriction enzyme digestion and agarose gel electrophoresis of plasmid DNA (data not shown). To test for cotransfer of pXO12, transcipient DNA was subjected to PCR amplification using primers specific for Tn*4430* sequences on pXO12. An amplification product was obtained from every isolate tested, indicating 100% cotransfer of pXO12 with pBC16 (data not shown). To test for cotransfer of pXO1, transcipient DNA was subjected to PCR amplification using primers specific for the *atxA* gene of pXO1. No evidence for the presence of pXO1 in transcripients was obtained (Fig. 4, bottom).

In conclusion, analysis of the transcipient isolates confirmed the transfer of plasmid pBC16 and fertility plasmid pXO12 between two *B. anthracis* strains in the rhizosphere of grass plants in our model system. These results indicate the potential for genetic exchange in the rhizosphere while providing additional indirect evidence for the existence of vegetative *B. anthracis* in the plant-soil model.

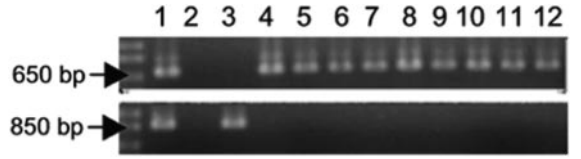

FIG. 4. Amplification of DNA sequences corresponding to the *tet* (top) and *atxA* (bottom) genes. Template DNA: lane 1, donor; lane 2, recipient; lane 3, 7702; lane 4, pBC16; lanes 5 to 12, transcipients.

DISCUSSION

Studies of *B. anthracis* have focused on the ability of the bacterium to cause anthrax, yet *B. anthracis* is related to common soil bacilli and is fully capable of surviving and multiplying outside of a mammalian host. Indeed, the bacterium is generally not directly transferred between hosts but is transmitted via contaminated soil and water (42, 59, 61, 62). Generally, the spore form of *B. anthracis* is considered to be the dominant form of the bacterium in the soil, and the capacity of vegetative bacilli to survive outside of the host has been rated as low and transient (59).

We designed a simple plant-soil model system to determine if *B. anthracis* spores germinate in the rhizosphere of plants. *B. cereus*, a close relative of *B. anthracis*, responds to germinants in the tomato rhizosphere (18). In preliminary experiments, we determined that *B. anthracis* spores can germinate on tomato seed coats and tomato plant roots (data not shown). Given that grasses are an important feed plant for large herbivores, we developed a system using tall fescue, one of the most abundant pasture grasses in the United States. This species is grown on millions of acres throughout the country. It is a perennial adapted to a wide range of soil and climatic conditions and is tolerant of continuous close grazing (48).

We used fluorescence of GFP-producing *B. anthracis* strains and Alexa Fluor 555-labeled anti-cell wall polysaccharide monoclonal antibody to confirm the presence of vegetative *B. anthracis* in the model system. Vegetative *B. anthracis* cells in the rhizosphere were apparent as filamentous chains and single rods up to 7 weeks following inoculation with spores. To further investigate the influence of the plants on the germination of spores, we compared heat-sensitive and heat-resistant CFU obtained from soil with and without seedlings. In the absence of seedlings, virtually all isolates recovered from soil at 2, 4, and 16 days postinoculation were heat resistant, indicating little if any germination. In contrast, up to 47% of the isolates recovered from soil containing seedlings were heat sensitive, indicating a significant amount of germination in the presence of the plant.

Accurate quantitation of germination and growth in the soil is complicated by a number of factors. One CFU can represent an individual cell or spore or multiple cells growing in a chain. Thus, in the case of *B. anthracis*, which grows in chains, vegetative-cell counts are likely to underestimate the number of viable bacteria. Moreover, the data obtained represent the steady state of viable bacteria. Germination and growth is likely countered by predation of *B. anthracis* by protozoans.

We occasionally noted ciliates with green-fluorescent food vacuoles. Also, cells may be lysed by bacteriophages. Streaks of most *B. anthracis* isolates from soil appeared to contain plaques. Lastly, the decrease in total CFU retrieved over time may also reflect an increase in the affinity of *B. anthracis* spores or cells for soil particles and plant roots over time (4).

Transfer of DNA between *B. anthracis* strains provided indirect evidence for the presence of metabolically active *B. anthracis* cells in the plant-soil system. Inter- and intraspecies plasmid transfer in the *B. cereus* group species is well documented for strains grown in batch culture (3, 25, 35, 47). In broth culture, coincubation of a donor strain harboring the fertility plasmid pXO12 and the tetracycline resistance plasmid pBC16 with a plasmid-free recipient strain can result in transfer of pBC16 at frequencies ranging from 1.6×10^{-4} to 7.1×10^{-2} transcipients per donor (25). In our model system, transfer of pBC16 was observed following incubation of *B. anthracis* donor and recipient strains in soil containing tall fescue seedlings. Transfer of pBC16 occurred at relatively low and variable frequencies. Nonetheless, our data indicate populations of metabolically active, vegetative *B. anthracis* cells present on and around the roots of grass seedlings in numbers sufficient for genetic exchange.

Horizontal gene transfer in the soil is important with regard to detection and identification of *B. anthracis* and the potential for evolution of new pathogens. Cotransfer of pXO1 with pBC16 from *B. anthracis* donor strains to various *B. cereus* group recipients in broth culture has been reported. In matings employing a $pXO12^+$ $pXO1^+$ $pBC16^+$ donor and selection for Tc^r transcipients, 0.3 to 0.4% of the $pBC16^+$ transcipients simultaneously acquire pXO1 (25). In our plant-soil system, preliminary attempts to select for transfer of a recombinant pXO1 carrying a selectable antibiotic resistance marker yielded no transcipients (data not shown). Nevertheless, further investigations of the potential for virulence plasmid transfer in the rhizosphere are warranted. The soil of outbreak sites is likely to contain not only *B. anthracis*, but other *B. cereus* group species.

In light of our findings, we suggest that investigations of so-called hot zones, areas in which anthrax epidemics in cattle and wild game occur regularly, should include sampling of roots and rhizosphere soil. Characterization of environmental isolates could contribute significantly to our interpretation of ambiguous clinical isolates. Currently, identification of *B. anthracis* is confirmed by the detection of pXO1 and pXO2. However, growth and genetic exchange of *B. anthracis* and related species in the context of the plant-soil environment may result in a number of ambiguous species, such as a recently discovered *B. cereus* strain that carries the anthrax toxin genes and was isolated from a patient with an anthrax-like illness (28).

Germination and growth of *B. anthracis* outside of the mammalian host is consistent with the lack of evidence for reductive evolution in *B. cereus* group species. Gene downsizing, gene deletion, and gene decay are considered hallmarks of microorganisms that are obligate pathogens or are otherwise limited in the ability to proliferate outside of a host (2). The recently published genome sequences of several *B. anthracis* (45, 46) and *B. cereus* (30, 44) strains do not suggest loss of genetic determinants which might be crucial for saprophytic survival.

The results presented here demonstrate for the first time that spores of *B. anthracis* can respond to germinants in the rhizosphere of plant seedlings in a manner similar to that of *B. cereus*. Our data suggest the possibility for a buildup of metabolically active, vegetative *B. anthracis* outside of the animal host with a potential for exchange of genetic material in the environment. Distribution of bacteria in the rhizosphere is not homogeneous. Spatial-distribution patterns are regulated by nutrient availability and competition by other rhizosphere inhabitants. Analysis of field samples for vegetative *B. anthracis* using nucleic acid-based detection methods is needed to further our understanding of evolutionary trends within the *B. cereus* group and to establish appropriate methodologies to assess potential risks associated with release of *Bacillus cereus* group species into the environment.

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